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Mapping of the Tacaribe Arenavirus Z-Protein Binding Sites on the L Protein Identified both Amino Acids within the Putative Polymerase Domain and a Region at the N Terminus of L That Are Critically Involved in Binding[▽]

Maximiliano Wilda,[1] Nora Lopez,[2] Juan Cruz Casabona,[2] and Maria T. Franze-Fernandez[1]*

Cátedra de Genética y Biología Molecular, FFyB, Universidad de Buenos Aires, Junín 956 (C1113AAD), Ciudad Autónoma de Buenos Aires, Argentina,[1] and Centro de Virología Animal (CEVAN), Instituto de Ciencia y Tecnología "Dr. Cesar Milstein," Consejo Nacional de Ciencia y Tecnología (CONICET), Saladillo 2468 (C1440FFX), Ciudad Autónoma de Buenos Aires, Argentina[2]

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Tacaribe virus (TacV) is the prototype of the New World group of arenaviruses. The TacV genome encodes four proteins: the nucleoprotein (N), the glycoprotein precursor, the polymerase (L), and a RING finger protein (Z). Using a reverse genetics system, we demonstrated that TacV N and L are sufficient to drive transcription and replication mediated by TacV-like RNAs and that Z is a powerful inhibitor of these processes (Lopez et al., J. Virol. 65:12241–12251, 2001). More recently, we provided the first evidence of an interaction between Z and L and showed that Z's inhibitory activity was dependent on its ability to bind to L (Jácamo et al., J. Virol. 77:10383–10393, 2003). In the present study, we mapped the TacV Z-binding sites on the 2,210-amino-acid L polymerase. To that end, we performed deletion analysis and point mutations of L and studied the Z-L interaction by coimmunoprecipitation with specific sera. We found that the C-terminal region of L was not essential for the interaction and identified two noncontiguous regions that were critical for binding: one at the N-terminus of L between residues 156 and 292 and a second one in the polymerase domain (domain III). The importance of domain III in binding was revealed by substitutions in D1188 and H1189 within motif A and in each residue of the conserved SDD sequence (residues 1328, 1329, and 1330) within motif C. Our results showed that of the substituted residues, only H1189 and D1329 appeared to be critically involved in binding Z.

Tacaribe virus (TacV) is the prototype of the New World group of arenaviruses. Within this group, the viruses form three phylogenetically distinct clades, one of which includes TacV together with the known South American pathogens that produce severe hemorrhagic disease: the Junin, Machupo, Guanarito, and Sabia viruses and the recently described Chapare virus (4, 7, 11). TacV, however, seems not to be a human pathogen.

TacV is an enveloped virus containing two single-stranded RNA segments called S and L. The S RNA contains two genes encoding respectively the nucleoprotein (N [64 kDa]) and the glycoprotein precursor (55 kDa) of the surface glycoproteins (13). The L RNA encodes the RNA-dependent RNA polymerase (RdRp) (L protein [240 kDa]) (16) and a small protein with a RING finger motif, called Z (11 kDa) (17). In both S and L RNAs, the genes are arranged in opposite orientations and are separated by noncoding sequences that have the potential to form stable secondary structures (14). S and L genomes and antigenomes are found only as nucleocapsids tightly bound to N protein, and the coding sequences are expressed from mRNAs transcribed from the 3′ region of the genomes and antigenomes (1, 14, 20, 27). These mRNAs end within the intergenic region and contain short stretches of nontemplated nucleotides at their 5′ ends, which are capped (20, 21).

Using a reconstituted transcription and replication system based on plasmid-supplied TacV RNAs and proteins, we demonstrated that L and N are the only viral proteins required for full-cycle RNA replication and transcription (22), for a faithful initiation of both processes, and for correct termination of mRNA transcription (21). Using this system, we also demonstrated that TacV Z protein is a potent inhibitor of both viral RNA replication and transcription (22). Lymphocytic choriomeningitis virus (LCMV) Z protein exhibits a similar inhibitory effect when viral RNA synthesis is analyzed both in the homologous (LCMV) reconstituted system and in the virus-infected cells (8, 9). Z protein is, in addition, essential for virus particle budding (24, 32) and has been proposed to play a regulatory role as it interacts with several cellular proteins (2, 3, 5, 19).

To understand the mechanism of Z-protein inhibition of viral transcription and replication, we investigated possible interactions between TacV Z and either of the proteins, L and N, essential for these processes. Using the TacV reconstituted system and coimmunoprecipitation of the plasmid-supplied viral proteins, we provided the first evidence that Z, at expression levels producing a potent inhibition of transcription and replication, interacted with L but not with N. This result, together with those derived from the contribution of different Z regions to its binding ability and inhibitory activity, strongly suggested that Z interferes with viral RNA synthesis by direct interaction with the L polymerase (18).

* Corresponding author. Mailing address: Cátedra de Genética y Biología Molecular, FFyB, Universidad de Buenos Aires, Junín 956, (C1113AAD) Ciudad Autónoma de Buenos Aires, Argentina. Phone and fax: (54-11) 4964-8296. E-mail: mtfranze@gmail.com.

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In this study, we mapped the Z-protein binding sites on TacV L protein. Using deletion analysis and site-directed mutagenesis, we identified amino acids within the putative RNA polymerase domain that are critically important for binding Z and defined a region at the N terminus of L that is essential for the interaction.

MATERIALS AND METHODS

Cells, virus, and sera. CV1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Invitrogen, Carlsbad, CA). Recombinant vaccinia virus vTF7-3, which expresses the T7 RNA polymerase, was kindly provided by B. Moss (National Institutes of Health, Bethesda, MD) (15). Monospecific sera against the recombinant TacV L were raised in rabbits as indicated before (22). Serum against glutathione *S*-transferase (GST) was obtained from GE Healthcare.

Plasmids. Plasmids pL, pgst, and pgstZ, expressing, respectively, the TacV L protein, the glutathione *S*-transferase (GST) protein, and the GST-tagged Z protein (gstZ), were described previously (18, 22).

Plasmids expressing the TacV L C-terminal deletion mutants L 1–1816 and L 1–1444 were generated by digestion with SmaI at the polylinker sequence together with either SnaBI (at nucleotide [nt] 1620 of the L genome) or PvuII (at nt 2740), respectively. For construction of the plasmid expressing L 1–1270, pL was digested with EcoRI at nt 3266 and at the polylinker sequence. In each case, the DNA fragment containing the truncated L gene was purified and religated, the stop codon being provided by the polylinker sequence of pL. To generate the plasmid expressing mutant L 1–1666, a PCR fragment was obtained from pL using a forward primer complementary to nt 2656 to 2636 of the L genome covering an SspI site and a reverse primer containing (5′ to 3′) an SmaI site followed by a stop codon and a sequence covering nt 2078 to 2093 of the L genome. The amplified fragment was digested with SspI and SmaI and inserted into pL digested with the same enzymes. Plasmids expressing the N-terminally truncated mutants L 156–2210 and L 292–2210 were constructed by the following procedure. PCR fragments were generated from pL using forward primers containing (5′ to 3′) a BamHI site followed by a start codon and a sequence complementary to nt 6604 to 6587 or to nt 6199 to 6188 of the L genome, respectively. In both cases, the reverse primer contained nt 6087 to 6101 of the L genome, including the StuI unique site. The amplification fragments were digested with BamHI and StuI, and each was inserted into pL, replacing the wild-type (wt) BamHI-StuI fragment. For the plasmid expressing mutant L 912–2210, the PCR fragment was generated using a forward primer containing a sequence complementary to nt 4339 to 4322 of the L genome preceded by (5′ to 3′) a BamHI site followed by a start codon and a reverse primer covering nt 3706 to 3725 of the L genome, including an AflII site. The amplification fragment was digested with BamHI and AflII and inserted into pL digested with the same enzymes.

Single-point mutations on pL were performed using the Quick Change PCR mutagenesis kit (Stratagene) with primers containing the mutated sequences. For construction of the plasmid expressing mutant L C2a (see the legend to Fig. 3), pL was excised with BstEII at nt 2404 and 3526 and the restriction fragment (encoding amino acids [aa] 1182 to 1556) was replaced by the corresponding BstEII-BstEII fragment carrying the substituted D1329 codon.

All plasmids were purified by Qiagen tip-100 (Qiagen Inc.,Valencia, CA). The introduced mutations, reversions, and inserted sequences were confirmed by automated dideoxynucleotide DNA sequencing (Macrogen, Korea).

Analysis of protein interactions by coimmunoprecipitation. Subconfluent monolayers of CV1 cells were infected with 3 to 5 PFU of vTF7-3 per cell and transfected using LipofectAMINE 2000 reagent (Invitrogen) as described before (18). The amount of plasmids added to approximately 4×10^5 cells was 100 ng of either L or each of the TacV L mutants and 200 ng of the plasmids expressing gstZ or GST. Labeling of the cells for 1 h at 5 h posttransfection with the addition of [^{35}S]methionine-cysteine mix (150 μCi/ml; NEG 772, NEN PerkinElmer) and preparation of cell lysates were performed as previously described (18). Aliquots corresponding to about 0.5×10^5 to 1×10^5 cells were immunoprecipitated with sera to L or to GST, and the immunoprecipitated proteins were resolved by two-step sodium dodecyl sulfate-polyacrylamide gel electrophoresis containing 8% and 12% polyacrylamide and visualized by autoradiography as indicated before (18). Quantification of the bands was performed on a PhosphorImager (Molecular Dynamics). Z binding refers to the amount of L, L mutants, and revertants coimmunoprecipitating with gstZ using serum to GST and normalized for protein expression determined by immunoprecipitation with serum to L. Z binding to L was taken as 100%.

RESULTS AND DISCUSSION

TacV L is a 2,210-aa protein. Sequence alignments between RdRps of a number of segmented and nonsegmented negative-stranded RNA viruses revealed conserved amino acids clustered into three domains along the length of the protein and joined by variable regions (23, 25, 26) (see Fig. 2A). Domains I and II, at the N terminus of L, are conserved only within the L proteins of arena- and bunyaviruses. Downstream from domain II, domain III exhibits six sequence motifs centered around amino acids invariant in all RdRps. Five of these motifs, named premotif A and motifs A, B, C, and D, revealed homology with motifs found in other well-characterized polymerases (25, 26); the sixth motif, named motif E, is conserved only within segmented negative-stranded RNA virus polymerases (23). An additional region conserved only in L proteins of arenaviruses (domain IV) has recently been identified (33). With the exception of domain III, the putative RNA polymerase, sequence analysis did not suggest any biological function for the other conserved domains of arenavirus L proteins.

To study Z-L interactions, we employed a previously described coimmunoprecipitation analysis using plasmid-expressed proteins (18). Z was expressed as a fusion protein with GST, as we have demonstrated before that the GST-tagged Z protein (gstZ) possesses identical binding ability to L and has the same inhibitory effect on viral RNA synthesis as Z (18). We have previously shown, in addition, that for Z (or gstZ) and L interaction coexpression of other viral components is not necessary and that for maximal interaction Z (or gstZ) and L must be coexpressed (see Fig. 2, 4, 7, and 8 in reference 18). We therefore studied Z-L interactions by coexpression of gstZ with either L or each of the L mutants, followed by immunoprecipitation of the expressed proteins with specific sera and analysis of the proteins by gel electrophoresis.

Effect of C-terminal deletions of the L protein in the interaction with Z. We started mapping the region(s) of TacV L involved in the interaction with Z by constructing mutants with a series of truncations in the C-terminal region. Mutants L 1–1816, L 1–1666, L 1–1444, and L 1–1270 had deletions of 394, 544, 766, and 940 aa, respectively, from the C-terminal end of L (Fig. 1A). To test the ability of the truncated proteins to interact with Z, cells were cotransfected with plasmids expressing gstZ and either L or each of the L mutant proteins. The cells were then labeled and lysed, and aliquots of the cell lysate were immunoprecipitated with monospecific serum against either GST or L. As a control, a similar procedure was performed with extracts from cells coexpressing GST and L. When the proteins immunoprecipitated with serum to L were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the results showed that each of the L mutants was synthesized at levels similar to wt L, each mutant exhibiting an appropriately increased mobility with the increasing size of the truncation (Fig. 1B, lower panel). Analysis of the proteins immunoprecipitated with serum to GST (upper panel) confirmed previous findings indicating that the interaction with L is mediated through the Z portion of the fusion protein (18) as L coimmunoprecipitated with gstZ but not with GST (lanes 1 and 6). The results also showed that deletion of the last 394 aa had almost no effect on binding as coimmunoprecipitation of L

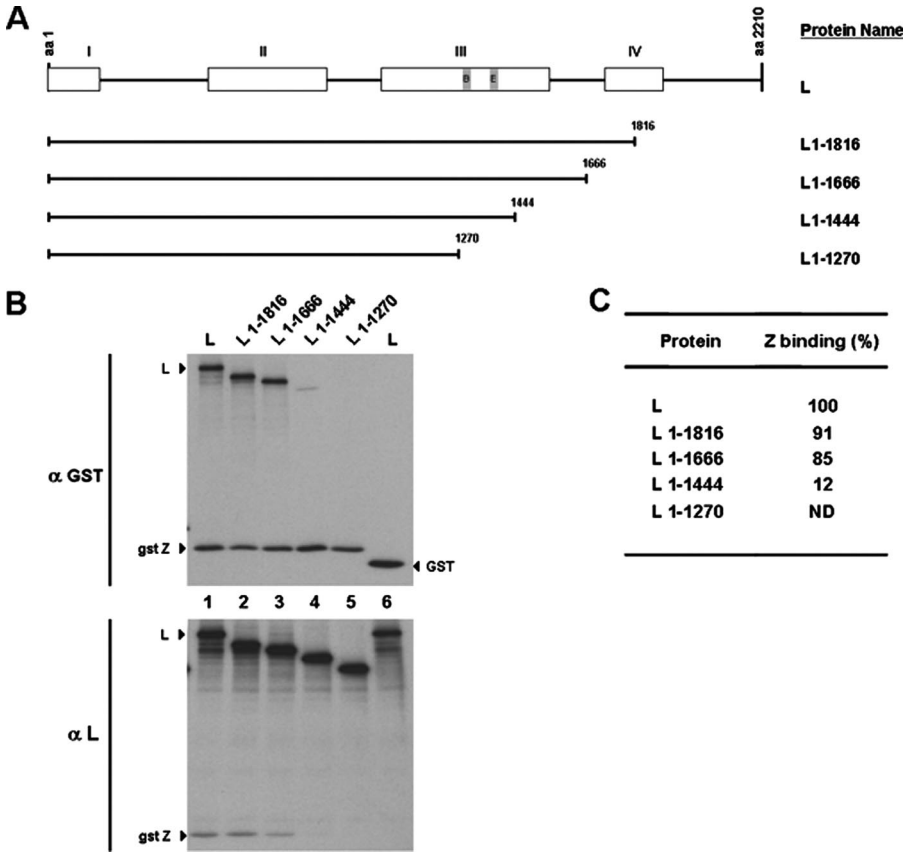


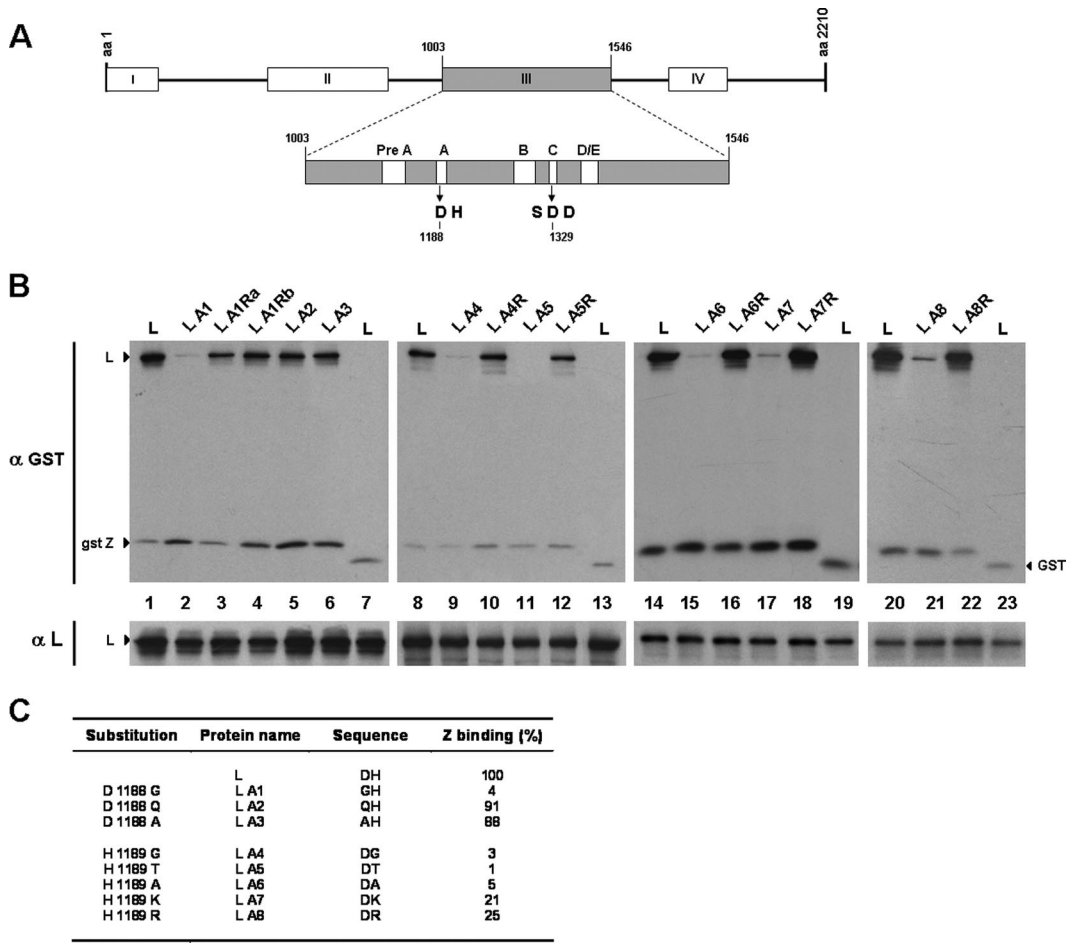
FIG. 1. Complex formation between Z and L proteins carrying C-terminal truncations. (A) Schematic representation of TacV L and of the C-terminal-deleted L mutants. The conserved domains I to IV and motifs B and E within domain III (25, 26, 33) are indicated. (B) Cell monolayers were transfected with either pL or each of the C-terminal mutant-expressing plasmids as indicated, together with pgstZ (lanes 1 to 5). As a control, pL was cotransfected with the GST-expressing plasmid (lane 6). Aliquots of cell lysates were immunoprecipitated using monospecific serum against either GST (αGST) or L (αL), and the immunoprecipitated proteins were resolved and visualized as described in Materials and Methods. The positions of L, gstZ, and GST proteins are indicated by arrowheads. (C) Z binding was calculated as described in Materials and Methods. The values represent the mean of three independent experiments; the relative variability intrasample ranged from 2 to 6%. ND, not detected.

1–1816 was more than 90% that of wt L. Even deletion of the last 544 aa in L 1–1666 gave nearly wt levels of binding (Fig. 1B, lanes 1, 2, and 3, and C). When deletion was extended to residue 1444, within domain III of L and approximately 50 aa downstream from motif E (see panel A), the resulting mutant protein (L 1–1444) retained only 12% the binding ability of L, whereas a further truncation to residue 1270, some 10 aa upstream from motif B, completely abolished the capacity of L to interact with Z (Fig. 1B, lanes 4 and 5, and C). It is worth noting that a similar pattern of interaction was observed when cell lysates were immunoprecipitated with serum to L (lower panel).

We also tested whether L proteins with deletions at the C-terminal end retained polymerase activity. For this, we used a previously described transcription and replication reconstituted system consisting of cotransfection of CV1 cells with plasmids expressing L, N, and a TacV RNA analog expressing the reporter gene chloramphenicol acetyltransferase (CAT) (22). We did not detect CAT activity when in the reconstituted system L was replaced by each of the C-terminally truncated L proteins (not shown). Thus, although the last C-terminal 544 aa of L were almost dispensable for its binding ability, they were essential for polymerase activity.

Identification of amino acids critical for binding Z within domain III of L. The above results indicating that C-terminal truncations extending within domain III of L impaired its ability for interacting with Z (Fig. 1), might suggest that residues within this domain are critically involved in binding. Alternatively, extensive C-terminal deletions might have caused conformational changes that altered the actual Z-binding sites at another region(s) of L. In favor of the first alternative was the observation that substitution of sequence motifs in domain III of TacV L by the homologous sequence motifs of the phylogenetically close Rift Valley fever virus L (33) abolished the capacity to interact with Z, although the TacV L mutants generated were full length (M. Wilda, unpublished).This prompted us to investigate an eventual role of domain III in L-Z interaction.

As domain III of TacV L is more than 500 aa in length, containing long stretches of sequences that are virtually completely conserved among all arenaviruses, we faced the challenge of selecting residues for site-directed mutagenesis. We



C

Substitution	Protein name	Sequence	Z binding (%)
	L	DH	100
D 1188 G	L A1	GH	4
D 1188 Q	L A2	QH	91
D 1188 A	L A3	AH	88
H 1189 G	L A4	DG	3
H 1189 T	L A5	DT	1
H 1189 A	L A6	DA	5
H 1189 K	L A7	DK	21
H 1189 R	L A8	DR	25

FIG. 2. Effect of amino acid substitutions in motif A of TacV L on interaction with Z. (A) Schematic representation of TacV L polymerase showing the pre-A to E motifs within domain III and the position of the strictly conserved D residues within motifs A and C. (B) Cell monolayers were cotransfected with pgstZ and either pL (lanes 1, 8, 14, and 20) or the plasmids expressing each of the single-point mutants as indicated (lanes 2, 5, 6, 9, 11, 15, 17, and 21). In lanes 7, 13, 19, and 23, pL was cotransfected with the GST-expressing plasmid. Aliquots of cell lysates were immunoprecipitated using monospecific serum against either GST (αGST) or L (αL), and the immunoprecipitated proteins were resolved and visualized as described in Materials and Methods. To control for amino acid substitution in mutants L A1, L A4, L A5, L A6, L A7, and L A8 being responsible for the low binding activity, at least one of the DNA clones expressing each of these mutants was changed back to the wt sequence using the Quick Change mutagenesis kit (Stratagene) with primers containing the wt sequence. Proteins expressed by the reverted plasmids (designated with the name of the corresponding mutant followed by "R") were tested for their binding activity as described above (lanes 3, 4, 10, 12, 16, 18, and 22). Revertants L A1Ra and L A1Rb were obtained from two independent DNA clones of L A1. The positions of L, gstZ, and GST proteins are indicated by arrowheads. (C) Z binding was calculated as described in Materials and Methods. Values correspond to the mean of two independent DNA clones of each mutant tested in three (L A2, L A5, and L A7) or in two (L A1, L A3, L A4, L A6, and L A8) independent experiments. The relative variability intrasample ranged from 1 to 11%. Z binding of revertants ranged from 78% to 100% that of wt L (not shown).

started modifying the invariant aspartate (D) 1188, around which is centered sequence motif A (Fig. 2A) (16, 23, 25, 26). We first replaced D1188 with G (mutant L A1) with the idea that the introduction of a residue that might affect the local environment in the region would help to identify sequences playing some role in binding. When lysates from cells coexpressing L A1 and gstZ were immunoprecipitated with serum to GST and the proteins were resolved by gel electrophoresis, it was found that coimmunoprecipitation of L A1 was severely affected, indicating that replacement of D1188 by G disrupted binding (Fig. 2B, compare lanes 1 and 2, and C). Low binding cannot be ascribed to instability of the protein as L A1 was full length and small differences in expression were normalized in order to estimate Z binding (see Materials and Methods). Moreover, as a control for the mutagenesis procedure, two independent L A1 clones were changed back to the wt amino acid and when the revertants (designated L A1Ra and L A1Rb) were tested for their binding ability to Z, each gave wt levels of binding (lanes 3 and 4). For a better understanding of the role of D1188 in binding, we engineered two other mutants in which either Q or A was substituted for D1188 (mutants L A2 and L A3, respectively). Each of these substitutions, however, had almost no adverse effect on binding (Fig. 2B, lanes 5 and 6, and C). Within motif A, we then selected histidine 1189 for site-directed mutagenesis as this amino acid is invariant in the L proteins of arenaviruses and changed this residue to five different amino acids (Fig. 2). When either G or T was substituted for H1189 (mutants L A4 and L A5, respectively) and the

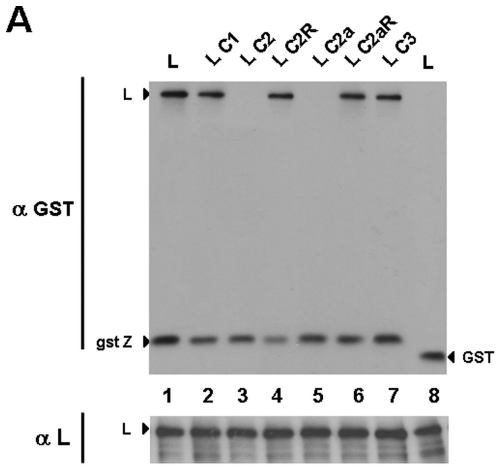
proteins were analyzed for their binding ability, each of these gave very low levels of interaction (Fig. 2B, compare lanes 9 and 11 with lane 8, and C). With the idea of exerting little effect in protein conformation (10, 12), we then replaced H1189 with A (mutant L A6). This change, however, also drastically impaired complex formation (Fig. 2B, compare lanes 14 and 15, and C). Finally, substitution of either K or R for H1189 resulted in five- to fourfold decrease in binding (mutants L A7 and L A8, Fig. 2B, lanes 17 and 21, and C). To control that low binding of the mutants was the consequence of amino acid substitutions, mutants L A4, L A5, L A6, L A7, and L A8 were changed back to the wt amino acid, and when the revertants were tested for their binding activity, each of them gave wt levels of binding (Fig. 2B, lanes 10, 12, 16, 18, and 22).

On the bases of sequence alignments, Muller et al. (23) transferred the structural information from human immunodeficiency virus reverse transcriptase to the putative polymerase domain of the negative-stranded RNA virus RdRps localizing the invariant D residues of motifs A and C within the palm region and close to each other. Considering that in TacV L the substitution of G for the invariant D1188 of motif A severely affected interaction with Z (Fig. 2), we expected to identify residues important for binding within motif C.

Motif C exhibits the SDD sequence conserved in segmented negative-stranded virus RdRps (Fig. 2A). The first D residue of the triplet (D1329) is strictly conserved in all classes of polymerases, whereas the second D is somewhat flexible as, i.e., it is replaced by N in nonsegmented negative-stranded virus RdRps (23). To identify residues within motif C playing some role in binding, we replaced each residue of the SDD triplet (positions 1328, 1329, and 1330) with A and the mutants generated (named L C1, L C2, and L C3, respectively) were analyzed for their binding ability. As seen in Fig. 3, substitution of A for S1328 (mutant L C1) or D1330 (mutant L C3) gave nearly wt levels of binding (Fig. 3A, compare lanes 2 and 7 with lane 1), whereas change of the invariant D1329 to A (mutant L C2) disrupted the interaction (compare lanes 1 and 3 in Fig. 3A). To control for whether or not the experimental approach used for the mutagenesis might have introduced systematic unnoticed mutations in L C2 that could affect its binding ability, we used a second experimental procedure to perform the substitution of A for D1329 (see Materials and Methods). The resulting mutant (named L C2a) also failed to interact with Z (compare lanes 1 and 5). Furthermore, both L C2 and L C2a were changed back to the wt amino acid and each of the revertants exhibited wt levels of binding (lanes 4 and 6). These controls confirmed that substitution of A for the invariant D1329 is responsible for the lack of binding ability of L.

We also tested whether mutations in motifs A and C affected polymerase activity. Using the TacV reconstituted system, we did not detect CAT expression above background levels when in the assay L was replaced by each of the L mutants depicted in Fig. 2 and 3 (not shown). These results confirmed and extended published data indicating that mutations in the invariant D of motif A or in the SDD triplet in motif C of LCMV-L generated nonfunctional proteins (28). A novel result is that H1189 in motif A is also strictly required for polymerase activity.

Deletion of the first 292 residues at the N terminus of L disrupts the interaction with Z. Although we have identified



B

Substitution	Protein name	Sequence	Z binding (%)
	L	SDD	100
S 1328 A	L C1	ADD	92
D 1329 A	L C2 / L C2a	SAD	ND
D 1330 A	L C3	SDA	93

FIG. 3. Effect of amino acid substitutions in motif C on interaction with Z. (A) Cell monolayers were transfected with pgstZ (lanes 1 to 7) or pgst (lane 8) and cotransfected with either pL (lanes 1 and 8) or the plasmids expressing each of the single-point mutants as indicated (lanes 2, 3, 5, and 7). L C2 and L C2a refer to the same mutant, differing in that their expressing plasmids were prepared by different procedures (see Materials and Methods). Aliquots of cell lysates were immunoprecipitated using monospecific serum against either GST (αGST) or L (αL), and the immunoprecipitated proteins were resolved and visualized as described in Materials and Methods. To control that the change of D1329 for A in mutant L C2/L C2a was responsible for the lack of binding, plasmids expressing each of these proteins were changed back to the wt sequence as indicated (Fig. 2 legend) and the proteins expressed by the reverted plasmids (designated L C2R and L C2aR) were tested for their binding activity as described above (lanes 4 and 6). The positions of the L, gstZ, and GST proteins are indicated by arrowheads. (B) Z binding was calculated as described in Materials and Methods. Values for L C1 and L C3 correspond to the mean of three independent clones of each mutant tested in three independent experiments in which the relative variability intrasample ranged from 4 to 6%. For L C2 and L C2a, the result is representative of five (L C2) and three (L C2a) independent clones tested in three independent experiments. The Z-binding capacity of the revertants (L C2R and L C2aR) ranged from 80% to 120% of wt L (not shown). ND, not detected.

residues within domain III of L that are critical for interaction with Z, we did not detect binding with mutant L 912–2210, which expressed the entire domain III plus the C-terminal region of L (Fig. 4A, B, lanes 1 and 2, and C). To define additional sequences that may be required for interaction, we engineered other N-terminal deletion mutants, of which L 156–2210 and L 292–2210 were stable (Fig. 4A and B, lanes 5 and 6, lower panel) and could be used to map the N terminus of L for binding. The results revealed that truncation of the first 155 aa did not significantly alter binding, whereas deletion of an additional 136 aa (mutant L 292–2210) disrupted the interaction (Fig. 4B, compare lanes 5 and 6 with lane 4, and C). These results led to identification of another region between residues 156 and 292 of L that is essential for binding Z.

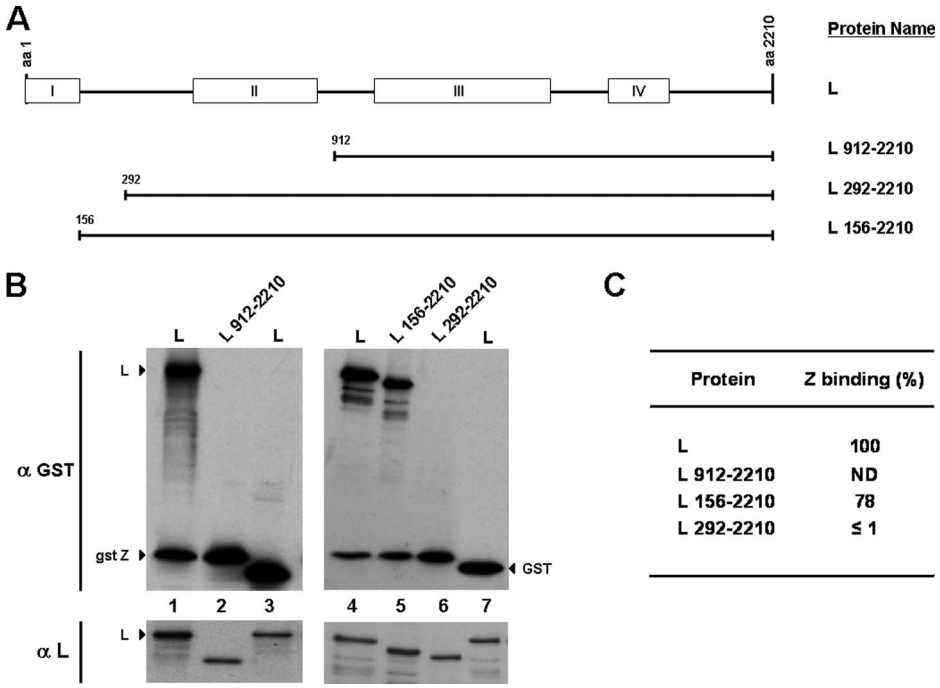


FIG. 4. Complex formation between Z and L proteins carrying N-terminal truncations. (A) Schematic of TacV L protein showing the conserved domains I to IV and the N-terminally deleted L mutants. (B) Cell monolayers were transfected with pgstZ (lanes 1, 2, 4, 5, and 6) or with pgst (lanes 3 and 7) together with either pL (lanes 1, 3, 4, and 7) or each of the N-terminal mutant-expressing plasmids (lanes 2, 5, and 6). Aliquots of cell lysates were immunoprecipitated using monospecific serum against either GST (αGST) or L (αL), and the immunoprecipitated proteins were resolved and visualized as described in Materials and Methods. The positions of the L, gstZ, and GST proteins are indicated by arrowheads. (C) Z binding was calculated as described in Materials and Methods. Results correspond to the mean of three independent experiments. For mutant L 292–2210, three DNA clones obtained from two independent cloning events were tested. The relative variability intrasample ranged from 1 to 4%. ND, not detected.

It is noteworthy that, like the L mutants with C-terminal deletions, none of the N-terminally truncated mutants exhibited polymerase activity when analyzed in the TacV reconstituted system (not shown). These results were not surprising since, at least in Sendai virus, L polymerase deletion of short sequences from the N or C termini abolish the functional activity of the protein (6, 30).

To the best of our knowledge, this is the first report identifying Z-protein binding sites on an arenavirus L polymerase. We have shown that the C-terminal region of L, including domain IV, seemed to be dispensable for the interaction and defined two noncontiguous regions that are critical for binding. The importance of domain III in binding was revealed by point mutations in sequence motifs A and C; each of these centered around an aspartic acid invariant in all polymerases. Within motif A, we changed both the invariant D1188 and H1189, the latter strictly conserved in arenavirus L proteins. Of the three substitutions for D1188, only change of D1188 to G impaired binding, whereas both the changes of D1188 to Q, with an uncharged polar side chain, and A, with a short hydrophobic side chain, were tolerated. On the contrary, substitution of either G, T, or A for H1189 reduced binding to 1% to 5% the wt level, and even the change of H1189 to K or R, with positively charged side chains, gave a five- to fourfold decrease in binding. Taken together, these results suggested that H1189 within motif A is critically important for the interaction with Z. We also performed Ala substitutions for each residue of the SDD sequence of motif C, finding that only the invariant first D of the triplet (D1329) appeared to be critical as its replacement with A completely abolished interaction, whereas the change of either S1328 or D1330 to A gave nearly wt levels of binding. Since motifs A and C have been proposed to be part of the catalytic site of the RdRps (23, 25, 26, 29, 31), it is a relevant finding that each of these motifs includes at least one amino acid critically involved in binding the inhibitory Z protein. In all probability, other residues in domain III play some role in binding as well. We are attempting their identification by means of Ala substitutions in conserved motifs within this domain.

Deletion analysis at the N terminus of L led to the definition of another region essential for binding Z between residues 156 and 292. Sequence alignments of this region between arenavirus L proteins revealed a low degree of homology (22%) and 10 invariant amino acids, which represent potential targets for mutagenesis and binding analysis.

Our finding that modifications in either domain III or in the N terminus of L impaired or ablated the interaction with Z could be interpreted to mean that both regions are required in concert for binding. Another possible explanation could be that one of the regions may contribute indirectly to the interaction: i.e., if integrity of the N terminus of L is essential for the overall conformation of the protein. In any case, it should be considered that the L proteins of a number of negative-stranded RNA viruses, including the arenavirus LCMV, may

act as a functional oligomer (see references 6 and the references therein and 28) and Z protein might require the oligomeric form of L for binding. It is noteworthy that the L-L interaction site on the L proteins of several nonsegmented negative-stranded RNA viruses maps at the N terminus of the proteins (see reference 6 and references therein). In addition, for L oligomerization (6, 28), as for L-Z binding (18), coexpression of the proteins is required. Taking these data together, it is tempting to speculate that modifications in the N terminus of L might prevent L oligomerization and in turn Z binding, as well. Efforts are under way to introduce modifications in the N-terminal region of L to map in detail the Z-binding site and to define the eventual involvement of this region in L oligomerization.

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